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Expression, detection of candidate function and homology modeling for *Vicia villosa* ornithine δ-aminotransferase

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Key words: proline, ornithine δ-aminotransferase, SDS-PAGE, bacterial transformation, homology modeling, salt tolerance

Abbreviations: δ-OAT, delta ornithine aminotransferase; BLAST, basic local alignment search tool; GPD1, glycerol-3-phosphate dehydrogenase 1; P5CS, pyrroline-5-carboxylate synthase; PAGE, polyacrylamid gel elecrtophresies; PDB, protein data bank; PRE, proline responsive elements; VvOAT, *Vicia villosa* ornithine aminotransferase

The accumulation of compatible solutes during stress in plant cell is well documented. Proline is one of these solutes that accumulates in the cytosol in response to drought or salinity stress in plants. Proline has several functions during stress just like osmotic adjustment, osmoprotection, free radical scavenger and antioxidant. Ornithine δ-aminotransferase (δ-OAT) is an important enzyme in proline biosynthetic pathway. It catalyzes the transamination of ornithine to pyrroline-5-carboxylate, which can be reduced into proline. Expression of ornithine δ-aminotransferase gene isolated from *Vicia villosa* (VvOAT) showed protein with a molecular mass of 63 KDa, which is compatible with the predicted mass and after VvOAT gene delivery into *E. coli* host HB101, VvOAT gene enhanced its salt tolerance. Homology modeling of VvOAT was performed based on the crystal structure of the ornithine δ-aminotransferase from humans (PDB code 2OATA). With this model, a flexible docking study with the substrate and inhibitors was performed. The results indicated that PHE170 and ASN171 in VvOAT are the important determinant residues in binding as they have strong hydrogen bonding contacts with the substrate and inhibitors. All the obtained results indicated the efficiency of utilizing this gene in conferring salt tolerance.

Introduction

All organisms, ranging from microbes to animals and plants, synthesize compatible solutes in response to osmotic stress.[1] Compatible solutes are non-toxic molecules such as amino acids, glycine betaine, glycerol, sugars and sugar alcohols. They do not interfere with normal metabolism and accumulate predominantly in the cytoplasm at high concentrations under osmotic stress.[2] These molecules may have a primary role of turgor maintenance, but they may also be involved in stabilizing proteins and cell structures.[3] Initially, it was thought that compatible solutes have their main role in osmotic adjustment, but there is increasing argument of other roles.[4] The accumulation of these solutes may not be important for osmotic stress tolerance but the metabolic pathways may have an adaptive value.[5] A further hypothesis is that compatible solutes are also involved in scavenging reactive oxygen species.[2,6-8]

Amino acid proline is one of the compatible solutes known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses.[9-15] In addition to its role as an osmolyte for osmotic adjustment, proline contributes to stabilizing sub-cellular structures (e.g., membranes and proteins), scavenging free radicals, and buffering cellular redox potential under stress conditions. It may also function as a protein compatible hydrotrope,[16] alleviating cytoplasmic acidosis and maintaining appropriate $NADP^+$/NADPH ratios compatible with metabolism.[17] Also, rapid breakdown of proline upon relief of stress may provide sufficient reducing agents that support mitochondrial oxidative phosphorylation and generation of ATP for recovery from stress and repairing of stress-induced damages.[17,18] Furthermore, proline is known to induce expression of salt stress responsive genes, which possess proline responsive elements (e.g., PRE, ACTCAT) in their promoters.[19,20] Ben Ahmed et al.[21] studied the ability of exogenous compatible solutes, such as proline, to counteract salt inhibitory effects in olive plants (*Olea europaea* L. cv. Chemlali) and found that the proline supplements seem to improve olive salt

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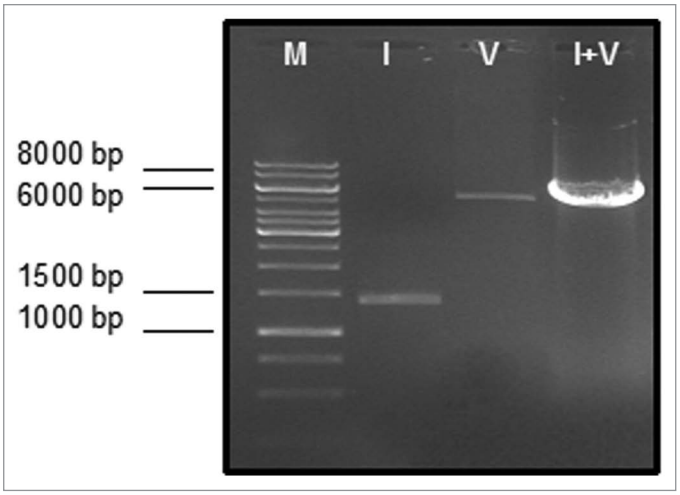

Figure 1. 1.5% agarose gel electrophoresis showing cloning of VvOAT into pET 30-b (M) Marker, GeneRuler™ 1 kb Fermentas, (I) OAT insert (1,410 bp) purified after digestion, (V) Purified pET-30b vector after digestion, 5,422 bp, (I + V) Digestion with *Hin*dIII of the new chimera p6X-VvOAT which contains both insert and vector, 6,832 bp.

tolerance by amelioration of some antioxidative enzyme activities, photosynthetic activity and so, plant growth and the preservation of a suitable plant water status under salinity conditions.

Ornithine δ-aminotransferase (δ-OAT) is an important enzyme in proline biosynthetic pathway. Overexpression of ornithine-δ aminotransferase (δ-OAT) represents an additional way to increase proline and enhancing of higher plants salt tolerance,[22] δ-OAT transaminates ornithine to pyrroline-5-carboxylate, which is further catalyzed to proline by pyrroline-5-carboxylate reductase.[23] The physiological role of δ-OAT is related to proline and arginine biosynthesis.

The glutamate pathway is thought to be the primary route for proline synthesis in plants during conditions of osmotic stress and nitrogen limitation whereas the ornithine pathway might function under high nitrogen input.[24] A study performed on NaCl-treated cotyledons of radish (*Raphanus sativus*) seedlings using the specific δ-OAT inhibitor gabaculine demonstrated the contribution of the ornithine pathway to proline synthesis.[25] In young *Arabidopsis thaliana* plantlets, free proline content, P5CS mRNA, δ-OAT activity and δ-OAT mRNA were all increased by salt-stress treatment.[26] Moreover, transgenic *Nicotiana plumbaginifolia* plants overexpressing δ-OAT from Arabidopsis synthesized more proline than the control plants and showed a higher biomass and a higher germination rate under osmotic stress conditions.[22] All these data suggest that the ornithine pathway, together with the glutamate pathway, plays an important role in proline accumulation during osmotic stress in plants. The aims of this study are to test the expression of the ornithine δ-aminotransferase gene isolated from the legume *Vicia villosa*, to detect its candidate function and to study the predicted tertiary structure of the protein encoded by this gene.

Results and Discussion

The ornithine δ-aminotransferase (δ-OAT) gene with accession number AF249857 cloned previously from *V. villosa* by Abdelhalim et al.[27] was subjected to gene expression examination. The pET System is the most powerful system yet developed for cloning and expressing recombinant proteins in *E. coli* and is now widely used because of its ability to mass-produce proteins.[28,29]

Novagen pET-30b vector was selected for expressing *V. villosa* ornithine δ-aminotransferase (VvOAT) gene into *E. coli* BL-21 (DE3). *Eco*Rl and *Sal*I double digestion applied to both pGEM T-eazy vector harboring the insert of VvOAT and native pET-30b vector. After recovery of both VvOAT insert and pET-30b vector (**Fig. 1**), ligation was applied to form the new recombinant plasmid p6X-VvOAT. Following ligation, recombinant plasmids were transformed into *E. coli DH5*α, plasmid DNA was isolated and subjected to screening for the presence of VvOAT by restriction digestion using *Hin*dIII enzyme (**Fig. 1**). After verification of the identity of positive clones carrying the gene, the construct was transformed into *E. coli* strain BL-21 (DE3) and prepared for 6X-VvOAT protein induction using IPTG.

The induction of BL-21 (DE3) cells containing the p6X-VvOAT plasmid using IPTG resulted in the accumulation of a recombinant protein with a molecular weight of 63 KDa in the cellular soluble fraction as revealed on polyacrylamide gel (**Fig. 2**). On the other hand, in the induction of BL-21 (DE3) cells containing the Native pET-30b plasmid, the band was not obtained. The best induction rate was obtained when using 1 mM IPTG for 3 hr. The predicted molecular mass of VvOAT is 51.3 KDa but as a fusion protein the molecular mass increased up to 63 KDa, which was obtained practically by SDS-PAGE. This result demonstrated that the isolated VvOAT gene sequence encodes for a fully intact protein, not truncated, and hence it was suitable to detect protein candidate function in prokaryotic *E. coli* after gene expression examination. Gafan et al.[30] expressed δ-OAT gene cloned from *P. falciparum* and obtained a subunit weight of 45 KDa on SDS-PAGE gels, which matched well with a theoretical weight of 46 KDa, Stránská et al.[31] expressed δ-OAT gene cloned from pea seedlings and obtained similar results, confirming the efficiency of expressing this gene in *E. coli*.

Detection of VvOAT gene candidate function was examined in *E. coli* strain HB101. After transformation of HB101 cells with native pET-30b vector and p6X-VvOAT, a single colony from each transformation was inoculated in LB medium supplemented with 0 mM NaCl, 700 mM NaCl, 800 mM NaCl and 900 mM NaCl in the presence of kanamycin and IPTG. Growth degree was determined after 24 h at an optical density of 600 nm. The results in **Figure 3 and Table 1** showed that the colony harboring the p6X-VvOAT plasmid had a higher OD value than the one with the native pET-30b vector throughout all the levels of salinity. This reflects that this *E. coli* colony acquired salt tolerance from the presence of induced VvOAT gene. Thus, the results indicated the ability of the gene in conferring salt tolerance. Edris et al.[32] transformed GPD1 gene (glycerol-3-phosphate

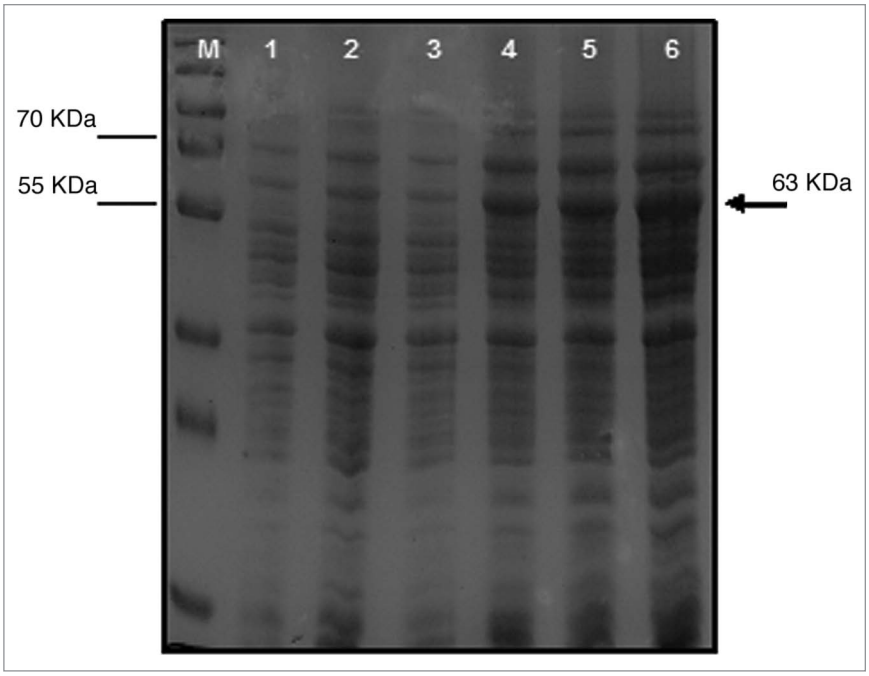

Figure 2. VvOAT expression in *E. coli*. All protein samples separated on 12.5% SDS-polyacrylamide gel. (M) PageRuler™ Plus prestained protein ladder, (1) Soluble fraction of BL21 cells containing native pET-30b plasmid (2) Soluble fraction of BL21 cells containing native pET-30b plasmid after induction with IPTG for 1 hr (3) Soluble fraction of BL21 cells containing chimera p6X-VvOAT plasmid, (4–6) Soluble fraction of BL21 cells containing chimera p6X-VvOAT plasmid after induction with IPTG for 1–3 hr, respectively.

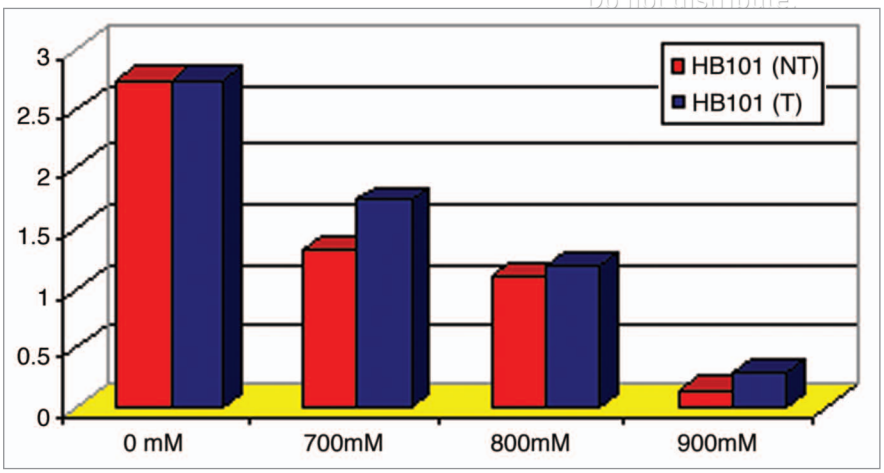

Figure 3. Effects of NaCl on transformed *E. coli* strain HB101 at concentrations of 700 mM NaCl, 800 mM NaCl and 900 mM NaCl.

dehydrogenase 1) from yeast *Saccharomyces cerevisiae* into three bacterial (*E. coli*) strains, DH5α, XL1 Blue and TOPO 10 and obtained different levels of tolerance due to the usage of different host strains. Expression of genes into *E. coli* might be a faster track towards knowing candidate function.

The results of BLAST search against PDB (2OATA) exhibited a high level of sequence identity (53.00%) to VvOAT. 2OATA, the crystallized structure of human ornithine,[33] was chosen as a reference structure for modeling VvOAT. The VvOAT 3D model (tertiary structure) was generated using ModWeb Server (**Fig. 4**). Comparative modeling strategy by Marti-Renom et al.[34] was employed in building and assessing this model. Structure-Structure alignment (**Fig. 5**) showed conserved secondary structure between target sequence VvOAT and template 2OATA.

Validation of the model was carried out using Ramachandran's plot. Calculations of the Phi and Psi distributions of nonglycine, non-proline residues are summarized in **Figure 6**. A percentage of 97.53% of the residues were in fully, additionally and generously allowed regions. This further indicated that the homology model is reliable. Hence, this model was used for docking the enzyme.

Docking was performed between the enzyme and its substrate ornithine and the enzyme with candidate inhibitors (serine, proline, valine and isoleucine). The results indicated that valine and isoleucine were competitive inhibitors for the binding of substrate ornithine because of the high energy of binding for both of them, which was -7.01 for valine and -7.41 for isoleucine (**Table 2**). On the other hand, serine and proline, showed a lesser energy of binding. It is also clear from **Table 1** that the binding of valine and isoleucine is stabilized by three hydrogen bonds while serine and proline are stabilized by only two. The previous results are agreed with Sekhar et al.[23] who made a docking study on *Vigna aconitifolia* δ-OAT and obtained similar results. Also from **Table 1** we can infer that PHE170 and ASN171 in VvOAT are anchored residues in binding as they have strong hydrogen bonding contacts with the substrate and inhibitors, but these results were not supported by Sekhar et al.[23] They reported that Gly106 and Lys256 in *Vigna aconitifolia* δ-OAT are the important residues for the binding of substrate and inhibitors.

Despite the advanced agrotechnologies, which are available these days, drought and salinity are still the major factors affecting crop yield by reducing growth and plant productivity. Ornithine δ-aminotransferase (δ-OAT) is an enzyme required for proline biosynthetic pathway that plays an important role in the adaptation of plants to soil salinity, drought and temperature stresses. Studying isolated δ-OAT from *Vicia villosa* enabled us to know more about its expression, candidate function and understanding the potential mechanism of enzyme and substrate binding and interactions between the enzyme and the inhibitors by using homology modeling and docking study. These studies were very important before the use of this gene in transforming

Table 1. Measures of the transformed and non-transformed *E. coli* strain HB101 culture turbidity

NaCl concentration	HB101 (NT) OD.	HB101 (T) OD.
0 mM	2.735	2.735
700 mM	1.325	1.735
800 mM	1.102	1.191
900 mM	0.156	0.286

Absorbance was determined at 600 nm after one day, (T = transformed and NT = non transformed).

Table 2. Docking VvOAT 3D model with its substrate and inhibitors

Total Intermolec. energy	Interacted residues with Hydrogen bonds	Docking
-9.56 kcal/mol	**PHE170 ASN171** GLU225 ILE227 LYS287 GLU225 THR262	OAT to Ornithin
-4.32 kcal/mol	PHE170 ASN171	OAT to Serine
-4.63 kcal/mol	PHE170 ASN171	OAT to Proline
-7.01 kcal/mol	PHE170 ASN171 GLN261	OAT to Valine
-7.41 kcal/mol	PHE170 ASN171 GLN261	OAT to Isoleucine

Bold text is determinant residues in binding.

economically important crops. However, it is important to trace δ-OAT function after gene delivery vis-à-vis the proline synthesis during exposure to salt stress.

Materials and Methods

Cloning of VvOAT into pET expression system. Previously isolated coding sequence of ornithine δ-aminotransferase from *Vicia villosa*[27] was cloned into pGEM-T easy vector (Promega, Madison, WI). The pGEM-T Easy vector harboring δ-OAT gene was double digested with *Eco*RI enzyme (located on pGEM-T easy vector) and *Sal*I enzyme (located on pGEM-T easy vector and after δ-OAT sequence). A fragment containing the gene was obtained and purified from agarose gel using AxyPrep™ DNA Gel Extraction Kit AP-GX-50. Plasmid pET-30b (Novagen) vector (found to contain suitable frame for VvOAT protein translation) was digested with the same enzymes and purified from agarose gel. Both purified insert and vector were ligated together using T4 DNA ligase and subsequently transformed into *E. coli DH5*α. After screening using *Hin*dIII enzyme new recombinant and native pET-30b plasmids were transformed into BL21(DE3) competent cells.

Expression of VvOAT into *E. coli*. Single colonies (one harboring cloned VvOAT in pET-30b and another harboring native pET 30b) were allowed to grow overnight on 5 ml LB supplemented with 50 μg/ml kanamycin. Bacterial cultures were diluted 1:10 with LB media and allowed to grow further for one hour then IPTG was added to a final concentration of 1 mM. One ml of bacteria was collected by centrifugation at 14,000 rpm for 1 minute at 4°C. Cultures were left to grow further for 4 hours after IPTG addition at 28°C. One ml of bacteria was collected by centrifugation at 14,000 rpm for 1 minute at 4°C after 1 h, 2 h and 3 h. Bacterial pellets were resuspended in 100 μl protein sample buffer and boiled for 5 minutes at 100°C. An aliquot of 15 μl was analyzed on 12.5% SDS-PAGE with pre-stained protein ladder Marker (PageRuler™ Plus Fermentas, Catg. # SM1811).

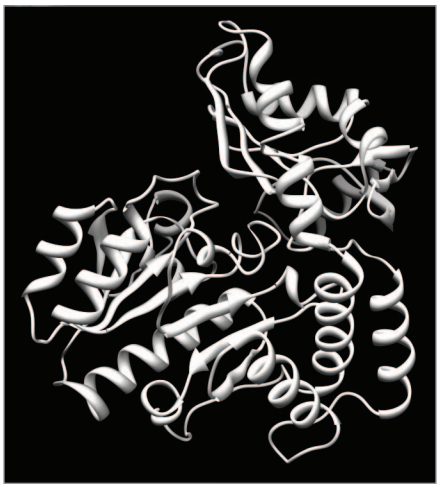

Figure 4. 3D Model of *Vicia villosa* ornithine δ-aminotransferase.

Detection of VvOAT candidate function. *E. coli* HB101 was transformed with pET-30b plasmid harboring δ-OAT gene and another transformation was made with native pET-30b plasmid. This was performed according to Edris et al.[32] with minor modifications. LB media (5 ml) tubes containing (0 mM), (700 mM), (800 mM) and (900 mM) NaCl (in addition to kanamycin 50 μg/ml and 20 mM IPTG) were inoculated with a single colony from individual cultures of the host strain carrying the plasmid pET-30b containing δ-OAT gene and host strain carrying the native pET-30b vector (control). Subsequently all inoculated cultures were incubated overnight at 37°C with shaking at 150 rpm. Growth degree was determined using optical density of 600 nm.

Homology modeling of VvOAT. The 3D model of VvOAT was built using ModWeb Server (modbase.compbio.ucsf.edu/scgi/modweb.cgi) A Server for Protein Structure Modeling,[35] by selecting the longest well-scoring model. The query sequence from *Vicia villosa* was searched to find out the related protein structure to be used as a template by the BLAST program,[36,37] against PDB. The one that showed the maximum identity with high score and less e-value was used as a reference structure to build a 3D model for VvOAT. Structure-Structure alignment,[38] and Ramachandran Plot map,[39] was created using web site (tardis.nibio.go.jp/joy/) and (dicsoft1.physics.iisc.ernet.in/rp/), respectively. Docking VvOAT enzyme 3D model with its substrate and inhibitors was performed using (www.dockingserver.com/web) to identify key residues in binding of substrate and inhibitors. Docking was made using default parameters except for the size of the simulation box (active site for docking) was 15 Angstroms, located at the center of the protein mass. PDB files of substrate and inhibitors were obtained from web data base (http://www.ebi.ac.uk).

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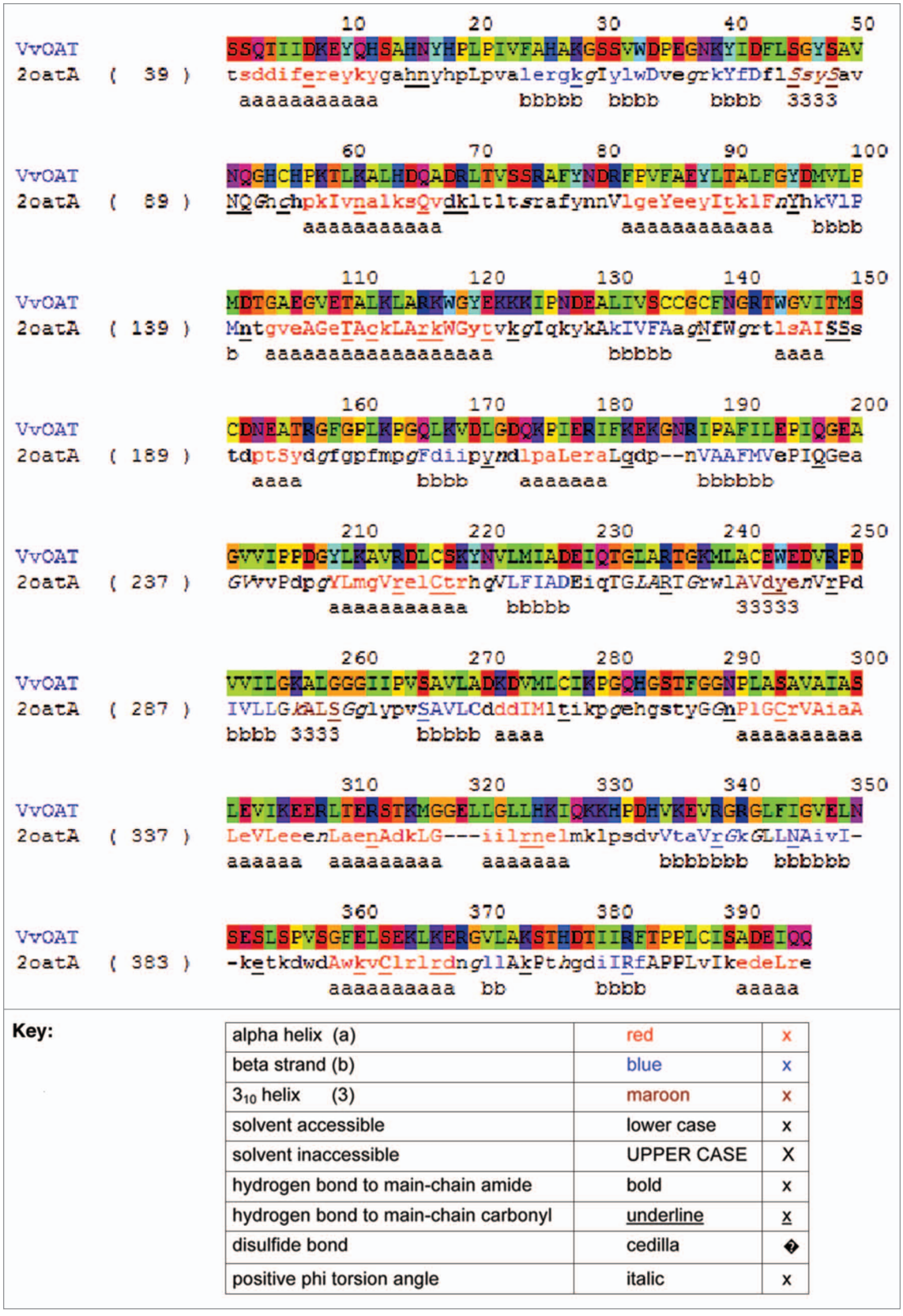

Figure 5. Structure-structure alignment of template and the final structure of VvOAT enzyme built using JOY server. The following table shows the key to the alignment.

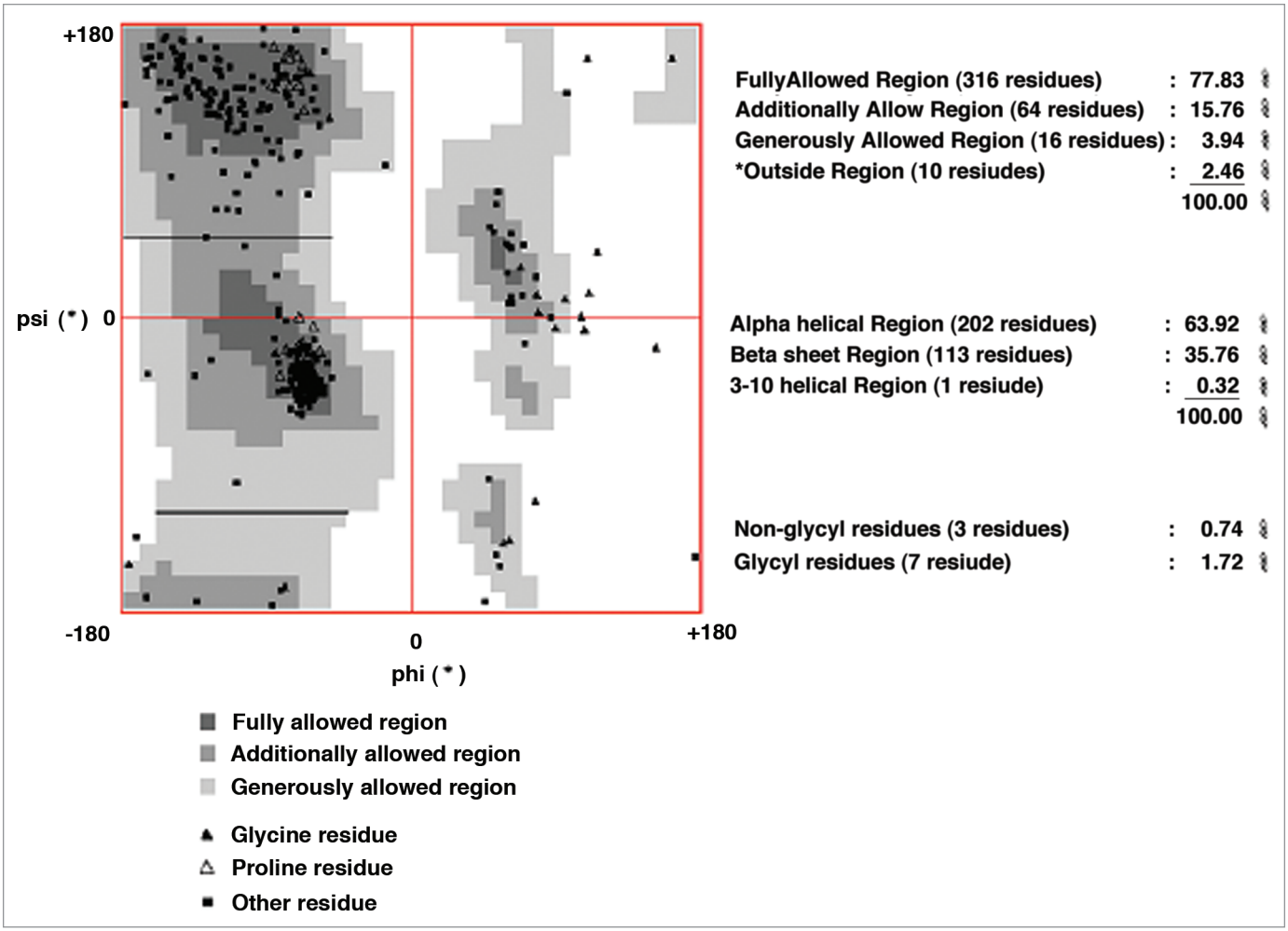

Figure 6. Ramachandran's map of VvOAT enzyme.

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